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14. ABSTRACT The Tulane/Xavier Biodefense Vaccine Development/Engineering project will develop new vaccines against biological threat agents to aid the war-fighter. Through the innovative use of nanotechnology, researchers and engineers from the Tulane University Schools of Medicine and Science & Engineering and the Xavier College of Pharmacy will fabricate nanoparticulate systems that are effective for transdermal and mucosal delivery of life-saving vaccines. One aim of this project will be to compare different nanocarriers (i.e., nanohydrogels, star copolymers, and spray-dried PLGA nanoparticles) for the ability to incorporate biological threat-relevant vaccine antigens and deliver those antigens through the stratum corneum to immune-responsive cells in the epidermis. The specialized assembly of each type of nanocarrier gives each unique properties and different interactions within the lipid channels of the stratum corneum. The use of nanocarriers for vaccine delivery is a platform technology, applicable to delivery of a variety of existing and potential vaccines.

15. SUBJECT TERMS
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INTRODUCTION

Infectious diseases remain one of the leading causes of death in adults and children world-wide. Each year, infectious diseases kill more than 17 million people, including 9 million children. In addition to suffering and death, infectious diseases impose an enormous financial burden on society. Although antibiotics and vaccines have been effective at reducing the morbidity and mortality of some infectious diseases, new ones such as AIDS, Lyme disease, West Nile fever, Hanta virus, SARS, and Avian Influenza virus are constantly emerging, while others such as malaria and tuberculosis reemerge in drug-resistant forms. Furthermore, we have an aging adult population with diminishing immune function, increased use of immunosuppressive agents for cancer, tissue transplantation, and autoimmune disease, and an upwardly spiraling cost of health care delivery that makes some existing vaccines unaffordable by the populations at greatest risk. In addition, we now face the possibility of bioterrorism with potentially devastating consequences and a limited number of preventative and therapeutic options.

A great deal of effort has been directed towards developing nonparenteral (needle-free) alternatives to traditional vaccine delivery. Nonparenteral vaccines offer a number of potential advantages over traditional vaccines including 1) the potential to confer mucosal as well as systemic immunity, 2) increased stability, 3) increased shelf-life, 4) elimination of needles and the need for specially trained healthcare specialists to administer vaccines, and 5) potentially lower costs. One such approach, transcutaneous immunization (TCI), is a non-invasive, safe method of delivering antigens directly onto bare skin. Immunization is achieved by direct topical application of a vaccine antigen. Despite the attractiveness of TCI, the technology is limited by the relative inefficiency of transport of large molecular weight vaccine antigens across intact skin.

Recent innovations in transdermal delivery of drugs, including chemical enhancers, electricity, ultrasound, and microneedles, demonstrate the feasibility of large-molecule transport through the skin's permeation-barrier, specifically the stratum corneum. This outer layer of the skin is composed of tightly packed lipid molecules and the dense, crystalline arrangement of these lipids creates the essential barrier to prevent water loss and pathogen entry. Recent evidence has shown that this barrier can be overcome by properly structured nano-sized particles (nanocarriers). This proposal will compare different nanocarriers for the ability to incorporate a model vaccine antigen and deliver that antigen through the stratum corneum to immune-responsive cells in the epidermis. The specialized assembly of each type of nanocarrier gives each unique properties and different interactions within the lipid channels of the stratum corneum. While the immediate objective will be to deliver vaccines against biological threat agents, the technologies created will have a tremendous impact on health and human welfare around the world because of their applicability to a wide range of infectious diseases and therapeutic treatments, including other infectious diseases that pose threats to the war-fighter and civilian populations.

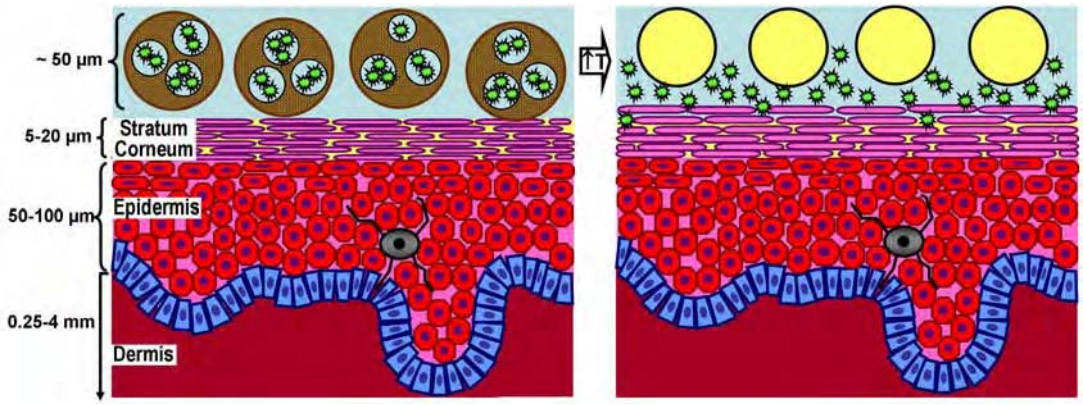
BODY

Through the innovative use of nanotechnology, researchers and engineers from the Tulane University Schools of Medicine and Science & Engineering and the Xavier College of Pharmacy will fabricate nanoparticulate systems that are effective for transdermal and mucosal delivery of life-saving vaccines. We will compares three different nanocarriers (nanohydrogels, star copolymers, and spray-dried PLGA nanoparticles) for the ability to incorporate a model vaccine antigen and deliver that antigen through the stratum corneum to immune-responsive cells in the epidermis. The specialized assembly of each type of nanocarrier gives each unique properties and different interactions within the lipid channels of the stratum corneum.

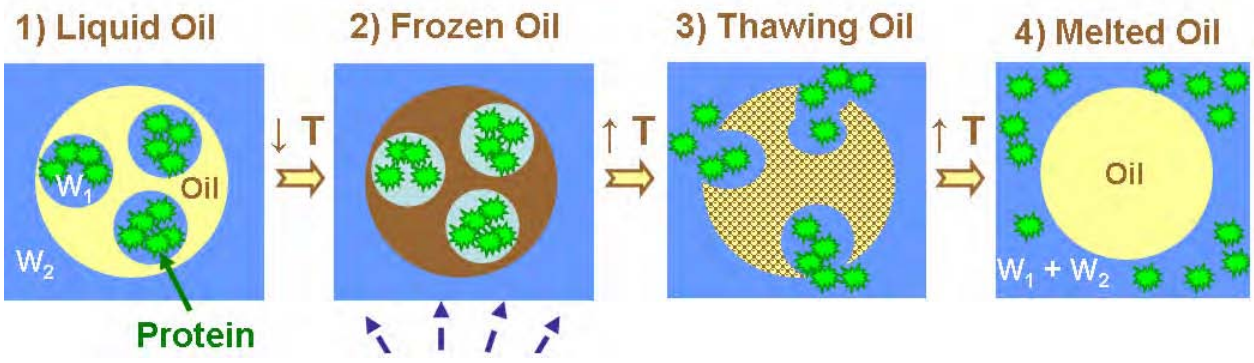
In the last funding cycle, we developed the analytical techniques to fully characterize permeation through the skin. We developed cryo-electron microscopy techniques using both cryo-scanning and cryo-transmission. We also fully implemented laser scanning confocal microscopy to understand time resolved permeation through the skin. We evaluated ceramide-3 and phosphotidylcholine containing liposomes, nanoscale amphipathic dendrimers, and double emulsions for their ability to deliver a protein antigen across intact skin. In the current funding cycle, we extend those findings on double emulsions, investigate nanoscale unimolecular reverse micelle (URM) carriers, and gel systems that are crystalline mesophases. Finally, we began immunization studies with BSA as a model antigen admixed with ceramide liposomes, water-in-oil-in-water (W1/O/W2) emulsions, tubular liposomes, and silica-tube nanogels.

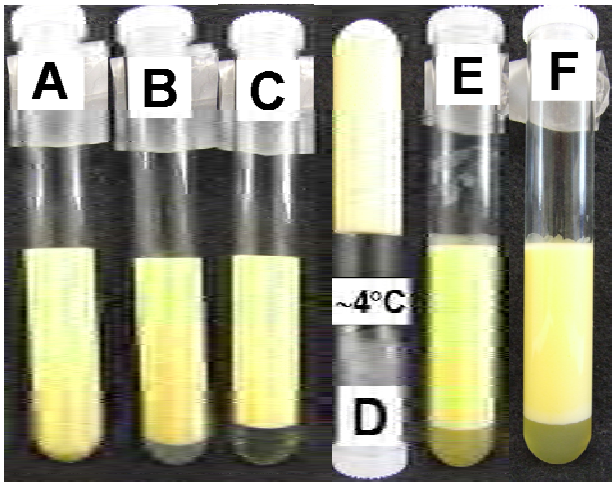
Double Emulsions

This part of our research consists of sheltering antigens within the safe microenvironment of the internal droplets of double emulsions for prolonged periods of storage, while at the same time enabling their easy release when applied onto a person's skin. The reason for this goal is that the rest of the formulation may contain agents such as "penetration enhancers," and other nanoparticles and chemicals with the function of harmlessly opening up the skin's pathways; such agents may denature the protein and strip it from its antigenicity. A scheme that will permit long-term storage and easy release foresees a water-in-oil-in-water (W1/O/W2) double emulsion with an oil phase that is solid at storage temperatures and thaws when brought to the temperature of the skin.



Our 2007 results from capillary video microscopy experiments had shown that when a stable double emulsion is prepared at a temperature where all three phases – W1, O, W2 – are liquid, and then is brought to a lower (storage) temperature where the oil phase freezes, stability is preserved. However, the crystallization of the oil causes "zone refining" of oil-soluble surfactants responsible for stability, so that when the double emulsion is brought back to temperatures where the oil phase thaws, instability takes place and the previously stored protein is now released. A schematic of this release mechanism is shown below



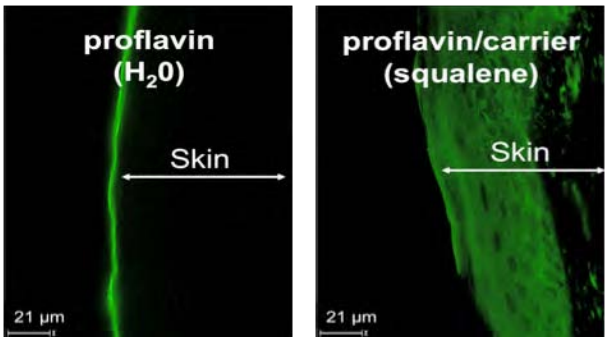


We then proceeded to formulate double-emulsions based creams that contain FITC-BSA in their internal W1 phase. A model W1/O/W2 double emulsion was prepared and subsequently subjected to temperature changes that caused the oil phase to freeze and thaw while the two aqueous phases remained liquid. As expected, no phase separation of the emulsion occurred if stored at temperatures below 18°C (freezing point of the model oil n-hexadecane) whereas oil thawing readily caused instability. Crucial variables were identified during experimentation and found to greatly influence the behavior of bulk double emulsions following freeze-thaw cycling. Adjustment of these variables accounted for a more efficient release of the encapsulated protein. In the figure below, we see various double-emulsion cream formulations, which demonstrate varying degrees of instability (phase separation). At 40C, the cream is totally stable.

Nanoscale unimolecular reverse micelle (URM) carriers

The goal of this research is to investigate whether discrete nanoscale unimolecular reverse micelle (URM) carriers can act as efficient carrier to bring polar therapeutics across the skin. The predominantly hydrophobic lipids of the stratum corneum act as an effective barrier keeping out most polar compounds, including polar drugs, peptides, proteins, and many potential antigens. The ability to encapsulate these bioactive molecules in “Trojan horse” nanocarriers—with a polar core for encapsulation, and a non-polar corona for skin compatibilization, offers an attractive route permeation enhancement. However, unlike traditional amphiphilic permeation enhancers, this “micellar” structure is covalent reinforced, and therefore their effectiveness cannot be disrupted by dilution, changes in polarity, etc.

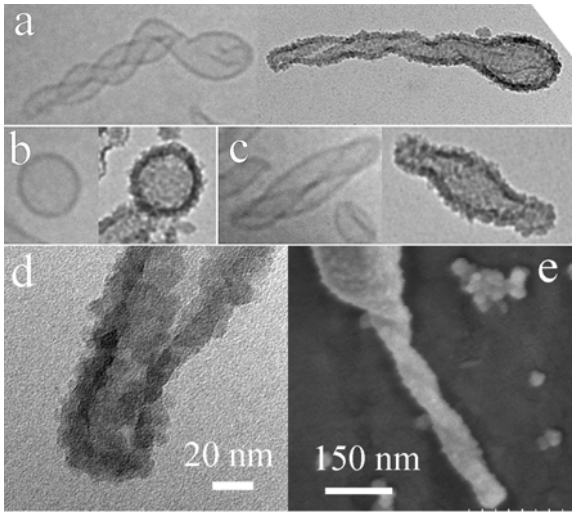
We have successfully prepared a family of star-block copolymers in which 3, 6, or 12 amphiphilic block copolymers arms are covalently tethered to a dendritic core. UV spectroscopic studies with polar dyes (e.g. proflavine) have verified these compounds’ ability to encapsulate and solvate small polar dye molecules in hydrophobic solvents in which they are not independently soluble. In comparison with analogous self-assembled micelles, the URMs demonstrate a higher encapsulation efficiency and their covalently reinforced structure is expected to have prevent disaggregation that may occur with self assembled systems. Fluorescent microscopy studies verify that they significantly enhance the transport of polar small molecules (proflavin dye) through porcine stratum corneum, which are otherwise insoluble in the lipids of the extracellular matrix. The inherent modularity of this system is expected to make this approach amenable to the encapsulation of significantly larger payloads, such as peptides and proteins, and these are presently under investigation.



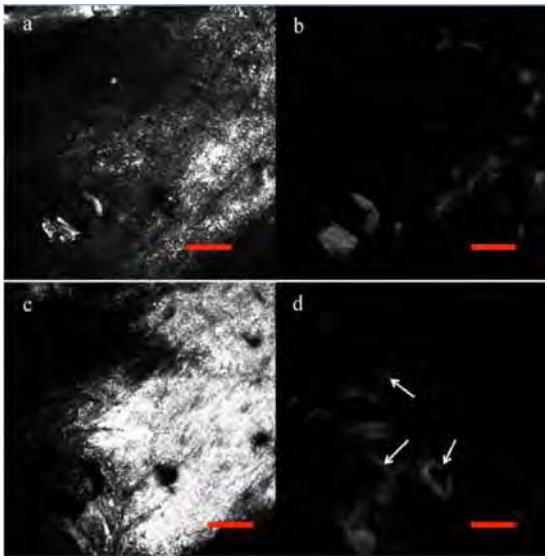
Gel systems that are crystalline mesophases

We have continued our previous research on the hydration dynamics of skin to understand lipid conformations. We have also conducted fundamental studies of gel systems that are crystalline

mesophases with the objective of incorporating antigens into the aqueous phase of the gel. During these studies, we developed high resolution NMR and cryo-electron microscopy techniques to characterize crystalline mesophases. The cryo-electron microscopy has also been of tremendous help to us in our studies of novel tubular liposomes created by the addition of ceramides to phospholipids. These tubular liposomes can be templated to ceramics. The next figure illustrates some of these liposomes imaged through cryo-transmission electron microscopy, and their ceramized counterparts.



From an applied perspective, we have developed very simple microneedles by coating ball bearings with powdered glass. By simply rolling these bearings onto skin, we create extremely small punctures that allow penetration into the stratum corneum. Below are images indicating penetration of indocyanine green. Tissue imaged using reflectance mode for the surface layer and 16 mm deep into porcine skin is shown in panels (a) and (c), respectively. Panels (b) and (d) show the penetration of indocyanine green at the skin surface and 16 mm deep, captured through fluorescence mode. Traces of indocyanine green are observed both on the surface layer and 16 mm deep into the skin. The arrows in panel d indicated penetration of fluorescent dye in the proximity of the perforations created. (Scale bar: 50 µm). These developments may lead to simple and inexpensive microneedle technologies for antigen delivery.



Transcutaneous Immunization

We have also initiated transcutaneous immunization studies with BSA admixed with different nanocarrier formulations. For these studies, Balb/C mice were immunized with 250 µg of BSA alone or admixed with (1) ceramide-3 liposomes, (2) W1/O/W2 double emulsions, (3) tubular liposomes, or (4) silica-tube nanogels. Duplicate groups included a transdermal adjuvant developed in our laboratories (LT(R192G)). The rationale for admixing rather than incorporating the antigen into the nanocarriers was to determine if the carriers themselves had an adjuvanting (as opposed to carrier) effect, whether there were any detrimental effects of the carrier/antigen ± adjuvant formulation, and (3) whether the nanoparticles could facilitate uptake of the target antigen without incorporation. We are in the process of analyzing the results at this time.

KEY RESEARCH ACCOMPLISHMENTS

- Using capillary video microscopy, we have shown that when a stable double emulsion is prepared at a temperature where all three phases – W_1, O, W_2 – are liquid, and then is brought to a lower (storage) temperature where the oil phase – O – freezes, stability is preserved. In this period we have extended our findings on double emulsions

- Investigated nanoscale unimolecular reverse micelle (URM) carriers, and gel systems that are crystalline mesophases.

- Began immunization studies with BSA as a model antigen admixed with ceramide liposomes, water-in-oil-in-water (W1/O/W2) emulsions, tubular liposomes, and silica-tube nanogels.

REPORTABLE OUTCOMES

E.C. Rojas, J.A. Staton, V.T. John, K. D. Papadopoulos. 2008. Temperature-induced protein release from water-in-oil-in-water double emulsions. Langmuir, 24, 7154-7160

Liu, L.; Tan, G.; Maskos, K.; McPherson, G.; John, V. 2008. High Resolution NMR Characterizations of Surfactant Crystalline Mesophases. Langmuir 20: 5301.

Tan, G.; Peng, X.; He, J.; McPherson, G.; Bose, A.; Agarwal, V.; John, V. 2008.Cryo-Field Emission Scanning Electron Microscopy Imaging of a Rigid Surfactant Mesophase. Langmuir 24: 10621.

CONSLUSIONS

Encapsulating a vaccine antigen within or adsorbing it to appropriate nanocarriers should facilitate transport through the stratum corneum to the targeted dendritic cells of the epidermis and dermis to initiate an immune response. Tailoring the nanocarriers to optimize encapsulation and/or adsorption and permeation efficiency requires an understanding of the interactions between the molecules composing the carrier, the antigen of interest, and the skin components in addition to the potential immune response to the antigen and the possible effect of the carrier or coadministered adjuvants on this response. Antigen-presenting cells show more efficient uptake of antigen incorporated into or onto a vesicular or particulate carrier, suggesting the potential for nanocarriers to enhance not only transport of the antigen through the skin's barrier but also uptake of the antigen once it reaches the dendritic cells of the viable epidermis and dermis. Nanocarrier-based transcutaneous vaccines represent a promising application of nanotechnology for delivery of vaccines against biological threat agents. Moreover, the technologies created will have a tremendous impact on health and human welfare around the world because of their applicability to a wide range of infectious diseases and therapeutic treatments, including other infectious diseases that pose threats to the war-fighter.